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Solid State Fermentation and Characterization of a Cellulase Enzyme System from *Aspergillus niger* SB-2

V. P. Zambare* and Lew P. Christopher

Center for Bioprocessing R&D, South Dakota School of Mines and Technology, 501 E. Saint Joseph Street, Rapid City, South Dakota, USA 57701, (*Email: Vasudeo.Zambare@sdsmt.edu; Phone: +1-605-877-2329)

ABSTRACT:

The focus of this study was on the solid state fermentation (SSF) of cellulase enzymes produced by *Aspergillus niger* SB-2 utilizing lignocellulosic agricultural waste as carbon and energy source. Optimization of the SSF media and parameters resulted in a 32% increase in the cellulase activity. Maximum enzyme production of 1,325±7.1 IU/g dry fermented substrate was observed on wheat bran and rice bran supplemented with malt dextrin and soybean meal at pH 6 and 30^0C after incubation for 120 h. The cellulase activities presented here appear to be among the highest reported in literature for *A. niger* to date. The *A. niger* SB-2 cellulase was partially purified and characterized. Zymogram analysis of the sodium dodecyl sulphate-polyacrylamide gel electrophoresis revealed two bands of cellulase activity with molecular weights of 30 and 45 kDa. To the best of our knowledge, a 45 kDa cellulase from *A. niger* has not been previously described in literature. The enzyme was active in a broad pH (4-7) and temperature (30-55^0C) range with a pH optimum of 6 and a temperature optimum of 45^0C. At 50 and 60^0C, the cellulase half life was 12.4 and 4.1 h, respectively. Dithiothreitol, iodoacetamide and Mg^{+2} acted as activators of cellulase activity. Kinetics studies indicated that the substrate specificity of *A. niger* SB-2 cellulase was 18% higher on insoluble cellulose than on soluble cellulose. Therefore, the cellulase complex of *A. niger* SB-2 would be useful in bioprocessing applications where efficient saccharification of lignocellulosic biomass is required.

Key Words: Agro-residues, *Aspergillus niger*, cellulase, media optimization, solid state fermentation

INTRODUCTION

Cellulases are among the biotechnologically important hydrolytic enzymes that are currently used in the food, feed and textile industries [1]. A recent focus of the research and development effort has been the application of cellulases in the enzymatic degradation of plant biomass to fermentable sugars for the production of biofuels such as bioethanol [2]. The complete enzymatic hydrolysis of cellulosic biomass requires the concerted action of three cellulase enzymes, namely endoglucanase (EC 3.2.1.4), exoglucanase EC (3.2.1.91) and β-glucosidase (EC 3.2.1.21) [3]. The production of cellulases and their properties have been extensively studied by various researchers [4-6]. The development of microbial strains, media composition and process control have all contributed to the achievement of high levels of extracellular cellulases [7] However, the cellulase costs are still too high for the establishment of a cost-effective production of cellulosic bioethanol [8].

As the feedstock costs contribute 20-50% of the total production costs, the use of readily available and inexpensive lignocellulosic biomass such as agricultural waste could certainly facilitate the commercialization of cellulose-based biofuels. Annually, several million tons of agri-wastes are generated world-wide including wheat bran, wheat straw, sugar cane baggase, rice husks, rice straw, rice bran, etc [9]. This waste is currently disposed of in landfills or burned out thereby imposing a negative impact on the environment. Through effective waste management, the agri-waste could be utilized in the production of value-added commodities and biofuels. However, the feedstock cost advantages of the biomass agri-waste waste may be largely negated by the high processing costs of cellulosic ethanol production (typically less than 10%) associated with the low ethanol yields and large amounts of water from the distillation process that need to be removed and treated. One approach to overcome these obstacles is to employ a solid state fermentation (SSF) process as an alternative method to the classical submerged fermentation (SF). The SSF process has the potential to significantly reduce the enzyme production costs and offers some apparent advantages over the SF such as increased product concentration, lower energy requirements, increased productivity, smaller effluent volumes and simpler fermentation equipment [10]. Many enzymes can be produced by SSF at a fraction of the cost of the SF method [11]. Other products of the SSF include pigments, pharmaceutical products, flavoring compounds and industrial chemicals [12]. The *in situ* produced solid-state enzymes can be applied without a prior downstream processing which further improves their competitiveness over the commercial liquid enzymes [13]. In addition, this approach would allow the development of a high solids simultaneous saccharification and fermentation process to reduce production costs associated with ethanol yield and productivity, equipment and water recovery costs, water availability and plant location.

Cellulases together with xylanases, pectinases and other lignocellulose-degrading enzymes are used by fungi for their growth in nature. *Trichoderma* spp. and *Aspergillus* spp. have most widely been studied and employed for commercial production of these enzymes [10]. Among the *Aspergillus* spp., *A. niger* has been repeatedly

reported as one of the most powerful cellulase producers capable of utilizing a variety of lignocellulosic substrates [14-18]. The SSF production of industrially important enzymes such as cellulase, ligninase, xylanase, pectinase, protease, amylase and glucoamylase by *A. niger* has been extensively described [10,19]. Work has been directed towards the optimization of fermentation media and processes of enzyme production [20,21]. This paper contributes to that effort. It examines the SSF production of a cellulase from *A. niger* SB-2 that utilizes readily available agro-residues. It further describes some of the biochemical properties of the *A. niger* cellulase in relation to its potential use in biomass bioprocessing to cellulosic ethanol.

MATERIALS AND METHODS

Microorganism and its maintenance

A mesophilic fungus *Aspergillus niger* SB-2 was isolated from garden soil sample collected from Nasik, India. It was propagated on Czapak Dox agar (CZA) medium (Difco, Germany). Slants were incubated at 30^0C for 5 days and stored at 4^0C.

Inoculum preparation

A 6 day-old slant culture was used to inoculate seed flasks with a medium containing 10 g wheat bran and 10 ml water [22]. After incubation at 30^0C for 6 days, the fermented medium was mixed aseptically with 50 ml of 0.1% Tween-80 saline for 30 min. Thereafter, the mixture was filtered through sterile glass wool and the spore count of filtrate was determined by serial dilution and spread plating of the filtrate [23].

Solid state fermentation

Rice husks, rice bran, wheat straw, wheat bran, sweet sorghum and baggase as agro-residues were collected from a local market and preserved at room temperature. These agro-residues (10 g) were distributed separately in 250 ml Erlenmeyer flasks, moisturized with 10 ml of water and sterilized at 121^0C for 30 min. Thereafter, 1 ml spore suspension (8×10^6 spores/ml) was added, flasks were mixed thoroughly and incubated stationary at 30^0C for 5 days. During the SSF of cellulase, the samples were removed aseptically at regular intervals of 24 h for up to 120 h of incubation.

Optimization studies

Cellulase production was optimized with respect to various environmental and nutritional parameters such as temperature (20-40^0C), moisturizing buffer pH (3-9), inoculum size (0.5-3%, v/w), initial moisture content (50-110%, v/w), nitrogen and carbon sources. The buffers used as moisturizing agents for pH control included 50 mM acetate buffer (pH 3-5), 50 mM citrate buffer (pH 6), 50 mM phosphate buffer (pH 7-8) and 50 mM Tris buffer (pH 9). The following inorganic nitrogen sources were used (0.25%, w/w): ammonium sulfate, ammonium phosphate, ammonium nitrate, sodium nitrate and urea. Yeast extract, peptone, tryptone, soybean meal, corn steep liquor (CSL), beef extract and malt extract served as organic nitrogen sources (1%, w/w). Glucose, malt dextrin, fructose, sucrose, lactose, starch, microcrystalline cellulose (MCC) and carboxymethylcellulose (CMC) were used at 1% (w/w) as supplementary carbon sources. The above chemicals and reagents were purchased from Himedia Labs (Mumbai, MH, India). Concentrations of nitrogen and carbon sources were further optimized by supplementing a wheat bran : rice bran (5 w/w : 5 w/w, 1:1) bed with 0.5-2% (w/w) soybean meal and 1-5% (w/w) malt dextrin. The incubation experiments (except the temperature experiment) were carried out at 30^0C for 120 h. All experiments were carried out in duplicate and data presented as the mean value± standard deviation (SD).

Cellulase extraction

Following SSF, 50 mM citrate buffer (pH-6) was added to the fermented dough in a 1:10 ratio (w/v) and homogenized with constant stirring at room temperature for 2 h. The suspension was filtered through Whatman filter paper no. 1 (Fisher Scientific, Pittsburgh, PA, USA) and the filtrate was centrifuged at 8371 x g for 15 min. The centrifuged supernatant was used as enzyme source for assay of cellulase activity.

Cellulase assay

The cellulase activity was determined by the assay method of Dutta et al. [24]. The supernatant containing cellulase enzymes (0.5 ml) was incubated with 0.5 ml of CMC (1%, w/v) in citrate buffer (50mM, pH 6.0) for 20 min at 45^0C. The reducing sugars released were measured with DNSA reagent using glucose as a standard [25]. One unit (IU) of cellulase activity was defined as the amount of enzyme that released one µmole of glucose equivalent per minute under the assay conditions. The enzyme activity was expressed as IU per gram dry fermented substrate (IU/gfs).

Fungal growth

Growth of fungal mycelia was estimated based on the amount of chitin present in the fungal cell walls [26]. The N-acetyl glucosamine, liberated upon acid hydrolysis of chitin, was mixed with 1 ml of acetyl acetone and incubated in a boiling water bath for 20 min. After cooling, the mixture was supplemented with 6 ml of ethanol and 1 ml of Ehrlich reagent, and incubated at 65^0C for 10 min. Thereafter, the optical density was read at 535 nm against a reagent blank and N-acetyl glucosamine (Himedia Labs, Mumbai, MH, India) as a standard. Fungal biomass was expressed as mg of N-acetyl glucosamine released per gram of dry fermented substrate (mg/gfs).

Partial purification and characterization of cellulase

The crude cellulase of *A. niger* SB-2 was partially purified by ammonium sulfate precipitation (60% saturation) at 4^0C and dialyzed against 50mM citrate

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buffer (pH 6). The partially purified enzyme was subjected to denaturation using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% (w/v) gels [27]. After electrophoresis, the gels were stained for protein with Comassie Brilliant Blue R250 [28]. Molecular weight standards (Sigma, St Louis, MO, USA) of α-lactalbumin (14.2 kDa), trypsin inhibitor (20 kDa), carbonic anhydrase (29 kDa), ovalbumin (45 kDa), albumin (66 kDa), and phosphorylase B (97 kDa) were used. Gel electrophoresis on 1% CMC (w/v) was run and analyzed by zymogram analysis [27]. The gels were stained for cellulase activity in a Congo Red solution (0.1%, w/v) at room temperature for 30 min. The activity band was observed as a clear colorless area, depleted of CMC, against a red background when destained in 1M NaCl solution.

The partially purified enzyme of *A. niger* SB-2 was characterized with respect to its activity under different pH (4-8) and temperature (30-60^{0}C) conditions. The thermostability of the enzyme was determined at 50^0 and 60^{0}C for up to 3 h. The effect of Na^+, K^+, Ca^{2+}, Hg^{2+}, Zn^{2+}, Fe^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Fe^{3+}, dithothreitol, and iodoacetamide on the enzyme activity was also studied. The substrate specificity of the partially purified cellulase was examined against MCC, avicel, CMC (Sigma, St Louis, MO, USA) and Whatman filter paper No. 1 at a substrate concentration of 10 mg/ml. The enzyme kinetic studies were performed with 1-10 mg CMC/ml according to Bharathiraja and Jayamuthunagai [29].

RESULTS AND DISCUSSION

Cellulase production on agro-residues

The SSF production of cellulase by *A. niger* SB-2 was carried out on various agro-residue substrates (rice husks, rice bran, wheat straw, wheat bran, sweet sorghum, and baggase). The use of rice bran alone and in combination with wheat straw (1:1) and with wheat bran (1:1) produced a peak in cellulase activity of 826±14.1 IU/gfs, 831±15.5 IU/gfs and 926±14.1 IU/gfs, respectively, after 5 days of fermentation (data not shown). Wheat bran alone and in combination with rice husks (1:1) also proved as an efficient inducer of cellulase with 768±5.4 IU/gfs and 795±5.6 IU/gfs, respectively. On the other hand, only 236±14.1 IU/gfs, 353±5.6 IU/gfs and 360±48.0 IU/gfs were produced on the single substrates of bagasse, wheat straw and rice husks, respectively. Our findings are in alignment with those of Chandra et al. [17] where wheat bran was reported as the best solid matrix for the SSF production of *A. niger* cellulase in a comparative study of several lignocellulosic substrates. Cellulase activities of 1.2 IU/gfs and 2.3 IU/gfs were produced in SSF of rice bran with *T. reesei* QM9414 and MCG77, respectively [30]. However, Kocher et al. [31] have shown that a strain of *A. harzianum* preferred rice husks as substrate for the production of cellulase. Therefore, the results reported here and elsewhere suggest that the cellulase enzymes are inducible and their substrate requirements in SSF may vary with the microorganisms used.

In our study, the cellulase production on rice and wheat bran correlated well with their nutritional content. Rice bran consists of 49.6% carbohydrates, 20.9% protein and 13.4% lipids as compared to 63.5-66.4% carbohydrates, 15.1-17.9% protein and 4.0-6.0% lipids in wheat bran (http://www.foodcomp.dk). Therefore, both rice and wheat bran contain significant amounts (15-21%) of protein which could be responsible for the induction of higher levels of cellulase during the enzyme biosynthesis in *A. niger*. Another factor which favors the enzyme production in SSF on these substrates is their relatively higher porosity than that of other substrates [32].

Effect of temperature on cellulase production

The temperature in a SSF system is determined from both the environment temperature and that generated from the metabolic activities of the growing fungi [33]. In our work, the cellulase production by *A. niger* SB-2 peaked (955±45.2 IU/gfs) at 30^{0}C (Fig. 1). Notably, the enzyme production declined at temperatures above and below 30^{0}C, with only 437±18.3 IU/gfs at 20^{0}C, and 95±5.6 IU/gfs, at 40^{0}C. It is known that the optimum temperature for enzyme production normally overlaps with the optimum growth temperature of *Aspergillus* sp. in its natural habitat [12]. Cellulase production and microbial growth by *A. niger* NCIM 1207 was optimal at 30^{0}C [15] while 28^{0}C was the optimum for the cellulase biosynthesis and cell growth in *T. harzianum* [4]. Maximum biomass and cellulase production was reported for *A. niger* at 35-40^{0}C by several researchers [34-36].

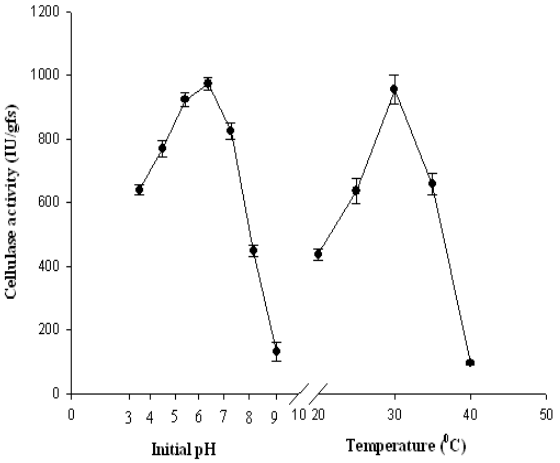

Fig. 1. Effect of initial pH (at 30^{0}C) and temperature (at initial pH of 6) on cellulase production by *A. niger* SB-2 on a wheat bran-rice bran (1:1) bed (100% moisture content, 1% inoculum, 120 h of incubation). Results represent the mean of duplicate determinations and bars indicate ± standard deviation.

Effect of pH on cellulase production

The SSF process is known to be influenced by the pH of the environment [37,38]. In confirmation, our results (Fig. 1) clearly indicate that the optimal production of cellulase by *A. niger* was pH-dependant. A maximum cellulase activity of 974±21.2 IU/gfs was obtained at initial pH of 6. The use of pH control to provide suitable

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conditions for optimum enzyme production in SSF was widely reported in literature. For instance, the thermoacidophilic fungus *A. terreus* M11 produced extracellular endoglucanase (581 IU/gfs substrate) on corn stover at pH 3 [39]. SSF of palm kernel meal for production of mannanase, cellulase and xylanase was carried out with several *Aspergillus* and *Trichoderma* strains at initial pH of 5 [40].

Effect of inoculum size on cellulase production

The effect of different inoculum sizes (0.5-3%, v/w) with initial spore count of 8 x10^6 spores/ml on the cellulase production by *A. niger* was studied for up to 5 days (Fig. 2). Generally, increased cellulase production is observed with increased inoculum size. For instance, Zhang et al. [41] reported that the increase of inoculum size up to 5% was beneficial for the cellulase synthesis by *T. viride*. In our study, we report a different behavior of *A. niger* where an increase in cellulase activity was only observed at inoculum size of up to 1% (v/w) and a further increase in the inoculum size did not influence the enzyme yield.

Effect of moisture content on cellulase production

The moisture content is an important parameter in the SSF process. Water activity affects the physical properties of the substrate mainly by causing swelling and facilitates effective microbial absorption of substrate's nutrients needed for microbial growth and metabolic activities [42]. As shown in Fig. 2, the gradual increase in the moisture content from 50% to 90% almost doubled the cellulase activity to reach a maximum of 977±18.3 IU/gfs. However, at moisture levels higher than 90%, the cellulase activity declined.

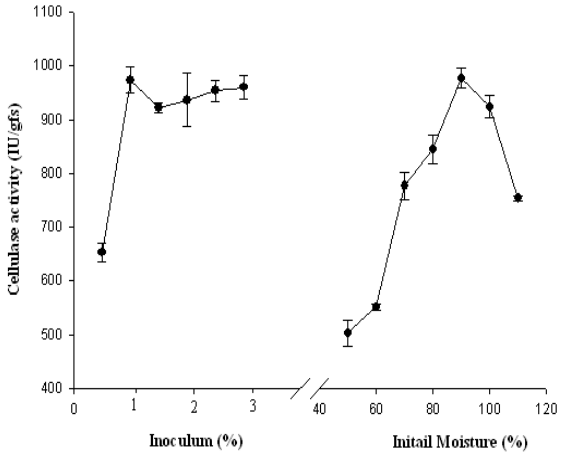

Fig. 2. Effect of inoculum size (at 100% initial moisture content) and initial moisture content (at 1% inoculum) on cellulase production by *A. niger* SB-2 on wheat bran-rice bran (1:1) bed (pH 6, 30^{0}C, 120 h of incubation). Results represent the mean of duplicate determinations and bars indicate ± standard deviation.

Generally, maximum cellulase production under SSF was reported to occur within the moisture content range of 60 to 70% [1,26]. A decrease in the water activity can cause partial enzyme adsorption to the substrate, due to decreased swelling and porosity of particles, resulting in substrate agglomeration [43]. Furthermore, low moisture levels can reduce gas volumes and impair gaseous diffusion that are the cause of poor aeration and low oxygen transfer [43,44].

Effect of nitrogen sources on cellulase production

The effect of nitrogen sources on the enzyme production by *A. niger* SB-2 in SSF is shown in Table 1.

Table 1. Effect of nitrogen sources on cellulase production[a] by *A. niger* SB-2.

Nitrogen sources	Cellulase activity (IU/gfs)[a]
Inorganic nitrogen sources (0.25%, w/w)	
Ammonium sulphate	793±4.2
Ammonium phosphate	370±14.1
Ammonium chloride	619±7.0
Ammonium nitrate	890±2.8
Sodium nitrate	765±21.2
Urea	926±14.1
Organic nitrogen sources (1%, w/w)	
Yeast extract	881±15.5
Peptone	1009±21.2
Tryptone	989±1.4
Soybean meal	1049±9.9
Corn steep liquor	685±14.1
Beef extract	657.5±12.0
Malt extract	585±1.4
Control (wheat bran-rice bran, 1:1)	963±33.9

[a] Solid-state fermentation conditions: wheat bran : rice bran (1:1) bed, pH 6, 30^{0}C, 90% (v/w) moisture, 1% (v/v) inoculum, 120 h of incubation.
[b] Values are the mean of duplicate determinations ± standard deviation.

Maximum cellulase activities were obtained with addition of soybean meal (1,049±9.8 IU/gfs) and peptone (1,009±21.2 IU/gfs). Heck et al. [45] reported the use of soybean waste residues for cellulase production by *Bacillus* sp. Likewise, Kocher et al. [31] described cellulase production in *T. harzianum* Rut-C 8230 with soybean meal. Addition of soya peptone to the growth medium improved the SSF production of cellulase by *T. lignorum* [14]. Thus, our findings on the positive impact of soybean meal and peptone during the SSF production of cellulase are in support of previous literature reports. The rest of the organic and inorganic nitrogen sources used in our work had no or minimal effect on cellulase production (Table 1). This however is in contrast to the work of Vyas et al. [46] who observed increased cellulase production by *A. terreus* in SSF using inorganic nitrogen sources.

Effect of carbon supplements on cellulase production

As poor growth in SSF systems is normally associated with the low nutritional capacity of the solid substrates, additional carbon-containing compounds are used in media formulations to enhance microbial growth. This subsequently results in improved production of primary metabolites including enzymes. In our study, the SSF production of cellulase by *A. niger* was examined in the

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presence of several carbon containing supplements (Table 2). These carbon supplements were added to the control carbon substrate (mixture of wheat bran and rice bran, 1:1) at 1% (w/w). When malt dextrin and MCC were added, the cellulase production increased by 266±14.1 U/gfs (29% increase) and 148±2.8 IU/gfs (16%) over the control, respectively. The other carbon supplements were less effective in enhancing the *A. niger* enzyme production. A cellulase production of 1,210±12.9 U/gfs (a 32% increase over control) was obtained with 2% (w/w) malt dextrin (data not shown). Maltose and dextrin were reported to enhance xylanase production in *Trichoderma* sp. FETLc3-2 [47].

Table 2. Effect of carbon containing supplements on cellulase production by *A. niger* SB-2.

Carbon sources (1%, w/w)	Cellulase activity (IU/gfs) [a]
Glucose	866± 22.6
Malt dextrin	1179±14.1
Fructose	780±4.2
Sucrose	843±18.3
Lactose	826±22.6
Starch	705±15.5
Microcrystalline cellulose	1061±2.8
Carboxymethyl cellulose	919±3.5
Control (wheat bran rice bran, 1:1)	913±16.9

[a] Solid state fermentation conditions: wheat bran : rice bran (1:1) bed, 1.5% soybean meal, pH 6, 30^{0}C, 90% (v/w) moisture, 1% (v/v) inoculum, 120 h of incubation.
[b] Values are the mean of duplicate determinations ± standard deviation

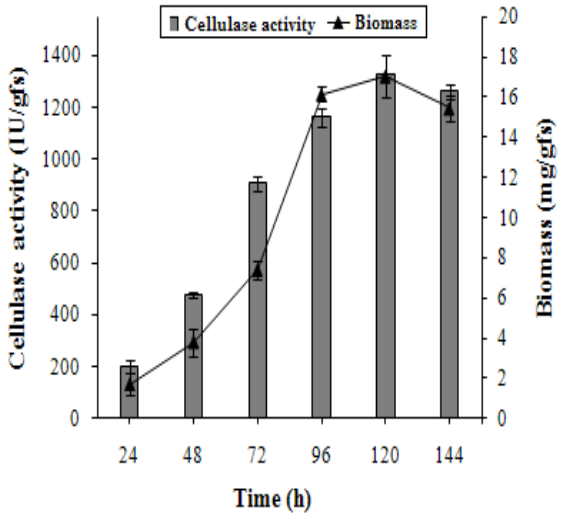

Fig. 3. Time course of biomass and cellulase production by *A. niger* SB-2 on wheat bran-rice bran (1:1) bed supplemented with 1.5% soybean meal and 2% malt dextrin (pH 6, 30^{0}C, 1% inoculum, 90% initial moisture content). Results represent the mean of duplicate determinations and bars indicate ± standard deviation.

Time course of cellulase production

A maximum cell growth (17.1±1 mg/gfs) and enzyme production (1,325±7.1 IU/gfs) occurred at 30^{0}C after 120 h of incubation under the following SSF conditions: wheat bran (5%, w/w), rice bran (5%, w/w), dextrin (2%, w/w), soybean meal (1.5%, w/w), 90% (v/w) moisture, pH 6 (50 mM citrate buffer), 1% (v/w) inoculum (8x10^6 spores/ml). The SFF of cellulase in *A. niger* SB-2 appeared to be growth-dependant and both cellulase and biomass production peaked on day 5 of incubation (Fig. 3). The thermoacidophilic fungus *A. terreus* M11 produced maximum cellulase activity of 581 U/g on the 5th day whereas the maximum biomass growth appeared on the 4th day of incubation [39]. It should be noted that the cellulase activity of 1,325 IU/gfs is one of the highest reported for *A. niger* to date (Fig. 3).

Enzyme characterization

The partially purified enzyme of *A. niger* SB-2 migrated on SDS-PAGE as several bands with different molecular weights (Fig. 4). Zymogram analysis of the crude enzyme revealed two bands staining for cellulase activity (C1, C2) where a clear hydrolytic activity zone was formed against a dark background. C1 and C2 migrated with molecular masses of 45 kDa and 30 kDa, respectively. Hurst et al. [34] reported a molecular weight of 26 kDa for *A. niger* cellulase. Isozymes of partially purified cellulases from *A. niger* (36 kDa and 23 kDa) and from *A. fumigates* (32 kDa and 21 kDa) were described [18]. High molecular weight cellulases (83 and 50 kDa) of *A. niger* were also reported [48]. Therefore, the cellulase enzyme system of *A. niger* appears to be composed of a number of proteins with different molecular weights [18]. Here, we report on a 45 kDa cellulase from *A. niger* which, to the best of our knowledge, has not been previously described.

The partially purified cellulase was active in the pH range 3-9 (optimum pH of 6) and temperature range 30-65ºC, with a temperature optimum of 45^{0}C (Fig. 5). Hurst et al. [34] published a report on a cellulase from *A. niger* which had an optimum pH and temperature of 4 and 45^{0}C, respectively. The cellulase of *T. viride* CMIT3.3 was found to be most active at 50^{0}C and pH of 4.8 [49]. An optimum temperature of 50ºC and a pH optimum of 6 were reported for purified cellulase enzymes from both *Aspergillus* sp. and *A. terreus* [50].

The *A. niger* SB-2 cellulase retained 85% of cellulase activity after incubation at 50ºC for 3 h (Fig. 6). The residual activities after 1 and 3 h were 99.5 and 84.6% (at 50^{0}C) and 86.4 and 49.6% (at 60^{0}C), respectively. The half-life of the enzyme was 12.4 (50^{o}C) and 4.1 h (60^{o}C). In comparison, Raj and Chandra [51] reported half-lives for an alkali-stable cellulase from *A. fischeri* Fxn1 of 4 h (at 50^{o}C) and 40 min (at 55^{o}C). A cellulase from *A. niger* Z10 retained 41.2% of its original activity when incubated at 90^{0}C for 15 min [48]. The half-life of a cellulase from the thermophilic bacterium *Thermomonospora* sp. was less than 1 h at 60^{0}C [52].

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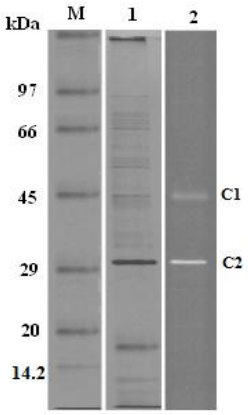

Fig. 4. SDS-PAGE of crude cellulase from *A. niger* SB-2. M, molecular weight markers: α-lactalbumin, 14.2 kDa; trypsin inhibitor, 20 kDa; carbonic anhydrase, 29 kDa; ovalbumin, 45 kDa; albumin, 66 kDa; phosphorylase B, 97kDa; 1, crude *A. niger* SB-2 cellulase; 2, zymogram of crude cellulase with carboxymethyl cellulose as substrate.

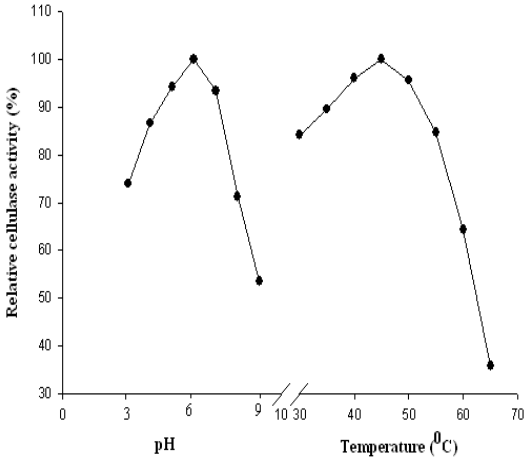

Fig. 5. Effect of pH (at 45^{0}C) and temperature (at pH 6) on activity of partially purified cellulase from *A. niger* SB-2 with carboxymethyl cellulose as substrate. Relative activity was expressed as percentage of maximum activity.

The influence of various metal ions and reagents on the cellulase activity of the partially purified enzyme from *A. niger* SB-2 was investigated (Table 3). A significant inhibitory effect on the cellulase activity was observed with Hg^{2+} (no activity) and Co^{2+} (5.6% relative activity). Other metal ions which had a negative impact on activity included Fe^{2+}, Ca^{2+}, Zn^{2+}, Fe^{3+}, Mn^{2+} and Cu^{2+}. On the other hand, the enzyme was significantly activated by dithothreitol (176%) and to a lesser extent by Mg^{2+} (115%) and iodoacetamide (110%). Dithothreitol and iodoacetamide have been reported to play an important role in the enzyme folding and activity of enzyme active sites [53].

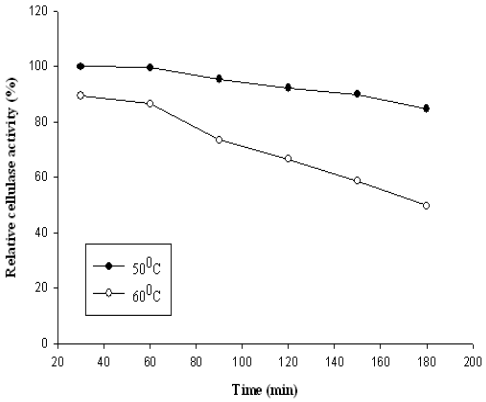

Fig. 6. Thermostability of partially purified cellulase from *A. niger* SB-2 (relative activity was expressed as percentage of maximum activity).

Table 3. Effect of metal ions and reagents on activity of a partially purified cellulase from *A. niger* SB-2.

Reagents (10 mM)	Relative cellulase activity (%)[a]
Control (without reagent)	100.0
Na^+	100.2
K^+	99.6
Hg^{2+}	0.0
Ca^{2+}	100.0
Zn^{2+}	89.6
Fe^{2+}	86.0
Co^{2+}	5.6
Cu^{2+}	16.3
Mg^{2+}	115.0
Mn^{2+}	21.0
Fe^{3+}	63.0
Dithothreitol	176.0
Iodoacetamide	110.0

[a] Relative activity was expressed as percentage of maximum activity.

The partially purified enzyme complex of *A. niger* SB-2 exhibited the greatest affinity for avicel followed by MCC, CMC and filter paper (data not shown). It was 18% more active on insoluble cellulose (avicel) than soluble cellulose (CMC). On CMC, the cellulase of *A. niger* SB-2 had K_m and V_{max} values of 2.6 mg/ml and 412 U/mg protein, respectively. The kinetic characteristics of β-glucosidases from several *Aspergillus* species were described [54-56]. Inoue et al. [57] reported that a cellulase of *Coptotermes formosanus* had a K_m of 1.9 mg/ml and a V_{max} of 148.2 U/mg protein on CMC. The K_m and V_{max} for endo-β-1,4-glucanase from *Gecarcoidea natalis* were 3.03 mg/ml CMC and 6.11 µmole/ml/min, respectively [58].

CONCLUSIONS

It was demonstrated that *Aspergillus niger* SB-2 has the potential to efficiently utilize lignocellulosic agricultural waste for production of cellulase enzymes. The cellulase activity of 1,325 IU/gfs obtained on a mixed substrate of wheat and rice bran is one of the highest reported for *A.*

niger to date. Partial purification of the cellulase complex of *A. niger* SB-2 revealed the presence of a new 45 kDa cellulase which has not been previously described in literature. The partially purified enzyme was 18% more active on insoluble crystalline cellulose than on soluble cellulose. This could have important implications of the *A. niger* SB-2 cellulase complex in the enzymatic breakdown of lignocellulosic biomass for the establishment of a robust and cost-efficient process for production of cellulosic ethanol.

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